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13. ABSTRACT (Maximum 200 words)

We are developing a novel degenerate cyclic peptide library selection method to identify potential peptide antagonists of the ErbB2 receptor tyrosine kinase. Since the aberrant activation of the ErbB2 receptor is believed to be involved in the genesis or progression of a significant proportion of human breast tumors, a cyclic peptide antagonist that binds selectively to the extracellular domain of ErbB2 could eventually be useful as an anti-breast cancer therapy. To identify potential antagonists, the extracellular ligand binding domain of the ErbB2 is immobilized on a column support, and used to affinity purify cyclic peptides from oriented random peptide libraries. The structures of oriented peptide libraries are based on the primary sequences of the twelve known peptide ligands for other members of the ErbB receptor family. Amino acid residues conserved in the known ligands are preserved, while those that vary are made degenerate at their corresponding positions in the library to select for high affinity binding to ErbB2. We anticipate that a high affinity synthetic peptide ligand for ErbB2 will be identified by this approach, which will then be assessed for its ability to act as an antagonist for the receptor.

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Introduction

A fundamental mechanism by which cancer cells promote their own growth and malignancy is through the aberrant expression of polypeptide growth factors. These factors are believed to act by binding to specific cell surface receptor tyrosine kinases that are expressed by the same or neighboring tumor cells, triggering pathways that stimulate cellular growth through autocrine or paracrine mechanisms. ErbB2 is a 185 kDa cell surface transmembrane receptor tyrosine kinase that can mediate the growth or differentiation of a variety of cultured cells, and contributes to the development of cardiac and neural tissues of developing embryos. Its overexpression in a number of human tumors, including breast and ovarian tumors, correlates with an earlier patient relapse and a poor prognosis. The observation that the overexpression of ErbB2 stimulates its protein tyrosine kinase activity, together with the observation that activated alleles of the *erbB2/neu* gene induce metastatic tumors when introduced into murine mammary epithelium, suggest that the aberrant activation of ErbB2 kinase activity plays an active role in breast tumorigenesis and progression. Its potential role in breast cancer, as well as its accessible localization at the surface of tumor cells, has made ErbB2 an attractive target for the development of agents that might disrupt breast tumor progression. This is illustrated with the recent approval of Herceptin, a humanized anti-ErbB2 monolclonal antibody that suppresses ErbB2 expression at the cell surface, by the Food and Drug Administration for administration to some breast cancer patients.

A predominant theme in signal transduction, the propagation of extracellular signals to intracellular structures such as the cytoskeleton or nucleus, is that specific protein-protein interactions are formed and dissociated during the signaling process. These interactions are often mediated by a relatively large domain on one protein that recognizes a relatively short peptide motif on another. For example, src homology-2 (SH2) domains consist of ~100 amino acids, and recognize specific sequences of 4-6 amino acids immediately surrounding a phosphorylated tyrosine residue. Likewise, protein kinase domains generally consist of 350-400 amino acids but recognize and phosphorylate short amino acid motifs of 4-8 residues. During my post-doctoral work in Dr. Lewis Cantley's lab, I was involved in the development of a novel method for identifying short peptide motifs that bind to specific domains of intracellular signaling proteins. The specific work in which I was involved concerned the identification of preferred substrates of protein kinases using oriented peptide libraries. However, the technique has been applied successfully in the determination of motifs recognized by a variety of domains contained within intracellular signaling proteins, including SH2, SH3, LIM, PTB and PDZ domains.

The object of the method is to present a library of possible ligands to the domain whose specificity is unknown, and to allow the domain to select the ligands to which it binds with high affinity. This is achieved by expressing, purifying to homogeneity, and immobilizing the domain of interest to a support and passing a library of peptides degenerate at several positions over this column. After washing, bound peptides may be eluted from the column and sequenced to determine which residues at the degenerate positions are selected by the domain. The key to making these kind of experiments work, however, is having some prior knowledge as to the kinds of motifs that the domain of interest prefers. If a particular residue is known to be part of the recognized motif, such as phosphotyrosine for SH2 domains, that residue may be introduced as non-degenerate in the library, and the surrounding residues left degenerate. While the non-degenerate residue(s) serves as a primary determinate for binding, the surrounding degenerate residues may be selected on the basis of the specific preferences of the particular domain.

In the studies described here, we extend this approach to examine peptide motifs selected by the extracellular domains of receptors, for the specific and novel purpose of identifying potential antagonists for the ErbB2 receptor. An antagonist of ErbB2 could act to prevent the auto-activation of the receptor associated with overexpression by interfering with its ability to homodimerize. Antagonists could also interfere with ErbB2 activation by blocking the binding of its intrinsic ligand, if such a molecule exists, or by blocking the heterodimerization of ErbB2 with other ErbB receptors stimulated by heterologous EGF-like ligands. The EGF-like ligands for the ErbB receptors have a common structure: six characteristically-spaced cysteine residues together with several other highly conserved or invariant residues. This points to the possibility that these ligands may serve as a foundation upon which to build oriented peptide libraries for identifying ligands for ErbB2. Such ligands can then be screened for their potential antagonistic properties.

<u>**Report**</u>

Specific Aim I: Express the extracellular domain of ErbB2 (ErbB2-ECD) in milligram quantities and purify the expressed product to homogeneity. The overall strategy for this aim is to simultaneously express and characterize the ErbB2-ECD in insect cells and mammlian cells and characterize the expressed products. From the characterization, the system that yields the most active protein in a form that is most readily purified will be up-scaled for production of recombinant protein. It is estimated that milligram quantities of purified protein may be obtained within one year.

A) *Transfection and expression of human ErbB2-ECD in insect cells and COS cells*
    1. *Subclone cDNA encoding the extracellular domain into expression vectors, sequence for PCR errors: months 1-3*
    2. *Transfection, selection of stably transfected COS cells and recombinant baculovirus, screen clones for ErbB2/Neu expression levels: months 3-5*
    3. *Characterization of the expressed products: months 6-7*
B) *Large scale production and purification of recombinant protein: months 8-12*

In months 1-12 of our studies we produced and purified the ErbB2 extracellular domain in High Five insect cells as a glutathione-S-transferase (GST) fusion protein using baculovirus technology. The cDNA encoding human ErbB2 extracellular domain was amplified by polymerase chain reaction, subcloned into the insect cell transfer vector pAcSecG2T, and confirmed by sequencing. [This vector encodes an insect leader sequence to target expressed proteins to the secretory pathway, fused to the GST protein. The coding region for the protein of interest is subcloned in-frame 3' to this, resulting in an amino-terminally-tagged fusion of the expressed protein with GST]. This material was used to generate recombinant baculovirus according to our standard protocols. Recombinant viruses were plaque purified, amplified and used to infect High Five insect cells to produce the recombinant protein. High Five cells are grown in the absence of serum, minimizing contamination from other proteins. This ~120 kDa fusion protein was recognized by five out of five tested conformationally-sensitive antibodies to the ErbB2 extracellular domain, strongly suggesting that the expressed protein was functionally similar to that found in breast tumor cells. Our studies indicated that we could obtain 150 μg protein per dish of High Five insect cells (20 mls of conditioned media). At that time we had assumed that the experiments of this specific aim had been successfully completed.

However, when characterizing the purification and library binding properties of this material we made two unexpected observations. First, we observed that although we were getting high level expression of our GST fusion in the conditioned media from the insect cells, we could only recover ~5% by affinity for immobilized GSH (reduced glutathione). The reason for the substantial loss of GSH binding activity is unclear, but may pertain to the fusion protein itself. We observed that the expressed secreted GST protein alone bound to GSH beads very efficiently. Hence, it is likely that the ErbB2 portion of the molecule folds back on the GST moiety to interfere with GSH binding. At any rate, the 95% loss translated into much larger scale purifications than we had previously anticipated. Preliminary attempts to separate our ~120 kDa expressed fusion protein from bovine serum albumin (we add 0.1 mg/ml BSA to the expression media as a carrier to avoid loss of the expressed protein at very low protein concentrations) by gel filtration and by ion exchange chromatography proved unsuccessful. More importantly, we also observed that GST itself binds a significant amount of our cyclic peptide library, making the signal-to-noise ratio in our experiments too low to observe spcific binding of the library to the ErbB2 extracellular domain (see below). For these two reasons

(together with the possibility that since the ErbB2 domain might be interfering with the properties of the GST domain, the GST moiety could interfere with the binding or selection properties of the ErbB domain), we were forced to re-engineer the ErbB2 extracellular domain construct.

For ease in purification, and to circumvent problems associated with the bulky 30 kDa GST moiety, we designed our second generation ErbB2 extracellular domain to be tagged at the carboxy terminus with six histidine residues. 6X His tags allow for rapid purification by affinity for immobilized nickel cation and elution using an imidazole gradient, and since antibodies to 6X His tags are available expression and purification may be monitored by immunoblotting. To build the construct we first subcloned the cDNA encoding the full-length human ErbB2 into the pVL1392 standard insect cell expression vector. We then replaced the DNA encoding the transmembrane and intracellular domains with a 600 bp PCR fragment encoding the carboxy-terminal half of the ErbB2 extracellular domain, where we designed the 6X His tag followed by a stop codon into the 3' amplifying primer. This construct, called His-2ECD, was transfected into Sf9 insect cells using the Bac-N-Blue transfection kit (Invitrogen), and recombinant viruses were plaque purified, amplified and used in subsequent infection of Hi5 cells for protein expression. We observed that the expressed protein migrated as a single band at ~ 95 kDa, the expected molecular weight, and we obtained ~50 μg of protein per 150 mm dish of cells (20 mls). This material could be immunoprecipitated by 7 monoclonal antibodies that are known to interact with human ErbB2 in a conformationally-dependent manner. Upon purification by nickel affinity we observed that the expressed protein would not significantly bind to the nickel column when the cleared conditioned media was put directly onto the column (Figure 1). However, when the cleared conditioned media was dialyzed 100,000 fold against PBS prior to loading, all of the expressed protein bound to the column and could be eluted with an imidazole gradient. Figure 2 shows a Coomassie Blue stain of a gel profiling a typical purification of His-2ECD. Prior to purification BSA is the predominant protein and His-2ECD is not detectable. However, we find that the expressed protein elutes with ~400 mM imidazole, and that His-2ECD is by far the predominant protein in the pooled fractions. While there is some prep-to-prep variation we estimate from these results that our purified material is 70-90% pure.

For our studies we must immobilize the His-2ECD protein and pass peptide library over the immobilized material for selection of high-affinity binders. The library is incubated with the bound protein, rapidly washed, and then bound peptides are eluted with either low or high pH. The mixture of eluted peptides is then sequenced to determine the optimal binding motif. One possibility for achieving immobilization is to covalently react the protein with a solid matrix such as activated agarose or Sepharose. The shortcoming of this approach is that the immobilization could sterically interfere with the binding of the peptide to its binding site. It would be optimal if we could anchor the protein at one of the termini such that the bulk of the protein is accessible for binding, and so that each receptor molecule is presented to the library in an identical manner. For the His-2ECD it is feasible to anchor the carboxy terminus to the nickel beads through the 6X His tag. However this does not covalently attach the protein to the support so that the His-2ECD could elute with the bound peptide at the pH extreme used to elute, and become the predominant component of the mixture that is ultimately sequenced. To test the possibility that we can carry out the library/His-2ECD binding reaction on the nickel beads we first examined the stability of the interaction of His-2ECD at two basic pH values that might be useful for eluting peptides. (Acidic conditions are known to disrupt polyHis/nickel ion interactions). Figure 3 shows that even at pH 11 His-2ECD remains stably associated with nickel beads, pointing to the possibility that the library binding and elution may be successfully carried out on this resin.

In summary, because our original ErbB2 extracellular domain construct was difficult to purify and its tag interfered with our assay, we were forced to re-engineer the construct and re-establish expression and purification strategies. Our new His-2ECD construct appears to be more well-behaved in that although it is expressed at a level 3-fold lower than our original construct, recovery with purification is 100%. The small 6X His tag significantly reduces the possibility that the library will non-specifically bind to the construct in a non-specific manner, and the 6X His tag affords a method of immobilization that should be superior to the covalent attachment of the construct to a resin.

Specific Aim II: Develop an oriented peptide library approach to determine peptide motifs selected by the ErbB2-ECD. On the basis of the structures of the known EGF-like ligands, a series of oriented peptide libraries will be designed, synthesized, characterized and employed in peptide selection studies to obtain the optimal motif for binding to ErbB2-ECD. The success of each of the libraries using this method cannot be predicted in advance, and the design of subsequent libraries will depend on results obtained from selection studies with previous ones. Of the three aims this is by far the most challenging, and it is anticipated that this step will require up to one and a half years.

A) *Synthesis, disulfide linking and characterization by mass spectrometry of first generation peptide libraries: may be carried out at the same time as receptor expression studies, months 1-12*

B) *Peptide selection studies and sequencing of first generation peptide libraries: months 13-14*

C) *Refinement of library designs from the results with first generation libraries, synthesis of new libraries, characterization, further peptide selection studies: months 15-30.*

Our first two synthesized libraries correspond to the third disufide loop of the active EGF-like ligands. The primary sequence of one library reads CXXXFXGXRC (called loop3), where X represents a mixture of 19 amino acids excluding cysteine, while the other reads CXXXFXGXRCXXX (called loop3lariat3). We attempted to oxidize this material under a variety of conditions and found that at low peptide concentration, efficient cyclization was achieved with minimal formation of library aggregates. Specifically, 20 mg of crude library made with a commercial peptide synthesizer was dissolved in 2 mls DMSO, and this was diluted dropwise into 100 mls 1M urea, 100 mM Tris, pH 8, 0.75 mM reduced glutathione, 1.5 mM oxidized glutathione, 100 mM methionine. This was stirred 48 hours at 4°C, dialyzed against deionized water 10^6 fold, and lyophilized. The resulting material was dissolved water. Figure 4 shows a mass spectrometry reading of loop3 at this point in the preparation. The broad peak centered at 1140 represents the library. The series of peaks in the 600-700 dalton range represent sodium adducts of oxidized glutathione which are inefficiently removed by dialysis with the 500 MW cutoff dialysis tubing employed. Non-cyclized material was removed using 5 mls immobilized iodoacetamide resin (Pharmacia), which covalently reacts with free sulfhydryl groups, and this material was dialyzed 10,000 fold against water using 1000 MW cutoff tubing. Figure 5 shows a mass spec reading of the resulting material. On the basis of these observations, we estimate that the libraries contain at least 70% cyclized, monomeric peptide.

We have examined the interaction of the loop3 library with the GST fusion of ErbB2 extracellular domain (GST-2ECD). In these experiments we expressed and purified either GST

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or GST-2ECD from the conditioned media of appropriately infected Hi5 insect cells, and independently immobilized 10 nmole of each protein on 1 ml of Affi-Gel 10 beads (Bio-Rad) according to the manufacturer's protocol. Unreacted moieties on the beads were blocked with 100 mM glycine, pH 8, and beads were washed with 10 mls PBS. Beads were then incubated with 1 mg (~1 µmole or 100 fold excess over target protein) loop3 library and incubated batchwise with rocking for 1 hour at room temperature. The mixture was poured into a 10 ml disposable column and the beads were rapidly washed (~5 seconds) with 10 mls ice-cold PBS. Bound peptides were eluted with 10 mM acetic acid, dried and subjected to sequencing by Edman degradation. At the same time an aliquot of the loop3 library was also sequenced for comparison with the eluted peptides to determine selection factors. From the cysteine content in the samples we were able to quantify the amount of peptide associated with each elution, because we defined the peptide library as containing 100% cysteine at two positions of the peptide and 0% at the remaining positions. We observed that similar levels of peptide were recovered from both the GST and and GST-2ECD columns(4.9 and 7.2 nmole respectively). A similar result was obtained when the loop3lariat3 peptide was used in the selection experiment. In subsequent experiments we observed little or no binding of peptide to resin that had no protein covalently bound to it, suggesting that the GST protein is capable of significantly binding our cyclic peptide library. Since 2/3 of the peptide bound to GST-2ECD is noise arising from GST binding, these data are difficult to interpret with confidence. For this reason the His-GST protein was generated (above).

Key Research Accomplishments, 1998-1999

- Carried out library selection experiments using GST-2ECD to select from loop3 library.

- Re-engineered ErbB2 extracellular domain construct to contain 6X His tag, His-2ECD.

- Expressed, purified and characterized His-2ECD protein for further selection experiments using loop3 and loop3lariat libraries.

Conclusions

In our first year we had prepared the initial two reagents necessary for the success of the project on schedule. We were able to produce and characterize two cyclic libraries based on the structure of the known EGF-like ligands. These libraries have the structures CXXXFXGXRC (loop3) and CXXXFXGXRCXXX (called loop3lariat3), where X represents a mixture of 19 amino acids excluding cysteine and the two defined cysteines in the peptide are disulfide linked. Separately, we were able to produce milligram quantities of homogeneous active ErbB2-ECD GST fusion by infection of High Five insect cells with recombinant baculovirus and purification of the recombinant protein by affinity for reduced glutathione. However, we observed that this material was difficult to purify. Additionally, two initial peptide library selection experiments suggested that the GST moiety was capable of binding significant quantities of cyclic peptide on its own. For these reasons we re-engineered the ErbB2 ECD construct to be 6X His-tagged at its carboxy terminus, and this product has now been characterized and is ready for use in selection experiments.

We are, however, encouraged by the results of these initial cyclic peptide selection experiments. The very low background observed with library incubated with beads alone, together with the observation that in the two experiments more peptide was recovered with GST-ECD than with GST alone, suggests that we were seeing specific binding. In the coming months we will carry out the selection experiment with loop3 and loop3lariat using the His-2ECD material. Since this protein does not carry a bulky tag that could itself interact with the library we anticipate a strong signal-to-noise ratio. These conditions should be optimal for the selection of high affinity binding of peptides. Such peptides will then be synthesized and their antagonistic properties characterized.

Figures

Figure 1. Expression of 6X His-tagged ErbB2 extracellular domain (His-2ECD). Hi5 insect cells were infected with recombinant baculovirus encoding nothing (wt), the carboxy-terminally His-tagged construct described above (His-2ECD), or a 6X His-tagged version of the extracellular domain of the unrelated receptor tyrosine kinase Tie2 (His-Tie2ECD) that migrates to a similar position. Conditioned media from these cells were resolved by 6% SDS-PAGE and blotted with anti-penta-His antibodies (Qiagen) to detect the His-tagged receptors (left three lanes, media). In the right three lanes, conditioned media was incubated with nickel beads, and bound proteins precipitated (nickel beads). These data demonstrate expression of the His-2ECD protein, and its poor binding to the nickel beads when presented in the conditioned media from insect cells.

Figure 2. Purification of His-2ECD. His-2ECD was expressed in Hi5 insect cells as described above. The conditioned media (~500 mls) was cleared by centrifugation at 100,000xg and the supernatant of this was dialyzed 100,000 fold against PBS and loaded onto 10 ml nickel resin. The resin was washed with 20 ml 10 mM imidazole, pH 7.5, 100 mM NaCl, and then eluted with 100 mls of an imidazole gradient (20 mM to 600 mM, pH 7.5, containing 100 mM NaCl). The column was then treated with 5 mM EDTA to remove nickel and all associated proteins. The purification was followed by blotting with the anti-penta-His antibody. Fractions containing His-2ECD were pooled and 100 ul analyzed by 6% SDS-PAGE followed by Coomassie blue staining. Lane 1, material prior to loading onto nickel column. Lane 2, 100 ul of 20 mM wash. Lane 3, pooled His-2ECD containing fractions. Lane 4, EDTA wash. These data illustrate the purification and the relative purity of the His-2ECD product.

Figure 3. Stability of the interaction of His-2ECD and nickel beads at high pH. Purified His-2ECD was adsorbed to nickel beads and split into two fractions. One fraction was treated for an hour at room temperature with 100 mM Tris, pH 8.8, while the other was treated with 100 mM triethylamine, pH 11. Beads were then pelleted and the pellets and supernatants analyzed by 6% SDS-PAGE followed by blotting with the anti-penta-His antibody. The left two lanes represent supernatants from the high pH incubation, while the right two lanes represent protein associated with beads. These data point to the potential utility of nickel bead-associated His-2ECD as a target in library selection experiments.

Figure 4. Mass spec of cyclized partially purified loop3 library. An aliquot of the sample described above was subjected to mass spectrometry to determine the extent of of monomeric and oligomeric library in the reaction mixture. The results suggested further purification was necessary.

Figure 5. Mass spec of final purified loop3 library product. An aliquot of the sample described above was subjected to mass spectrometry to determine the extent of of monomeric and oligomeric library in the reaction mixture. The results suggest that the library is pure cyclized material and at least 70% of it is monomeric. This material was used in the experiments described above and will be used in subsequent peptide selection experiments.
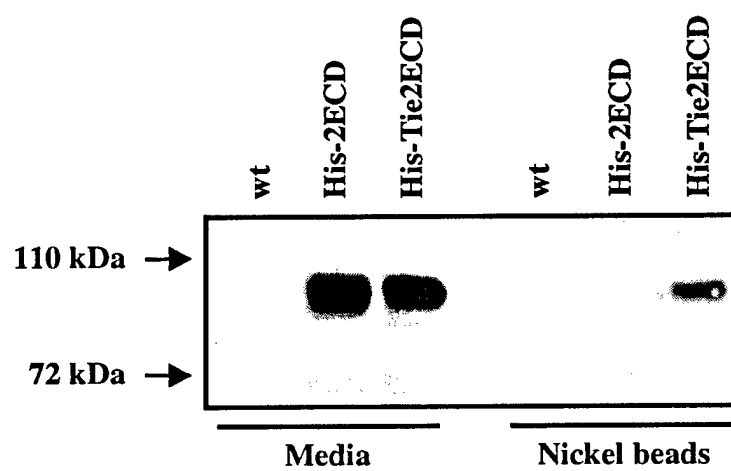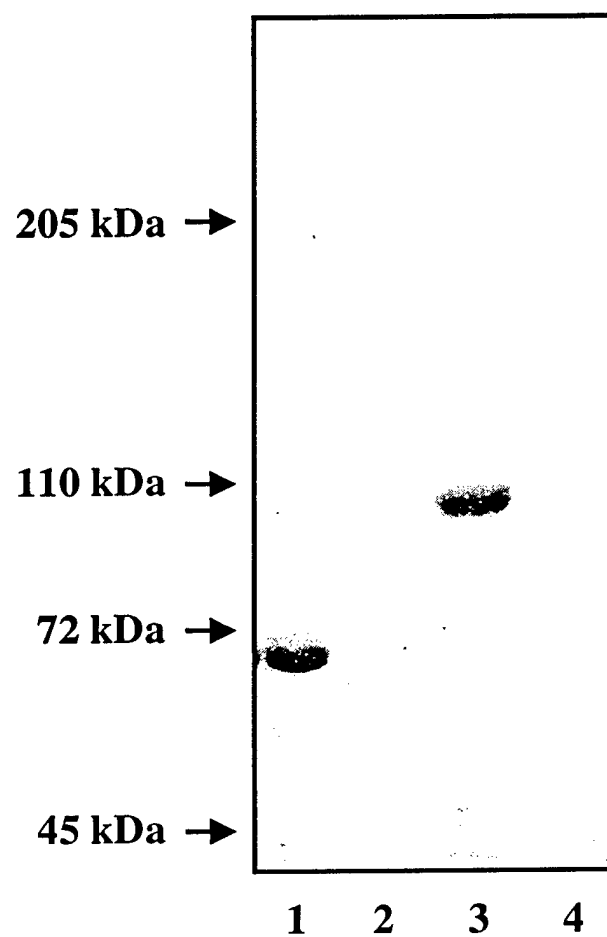
13

110 kDa →

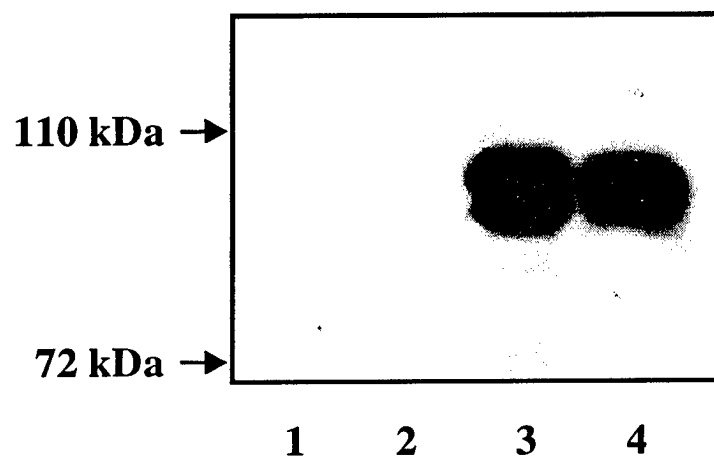72 kDa →

wt His-2ECD His-Tie2ECD wt His-2ECD His-Tie2ECD
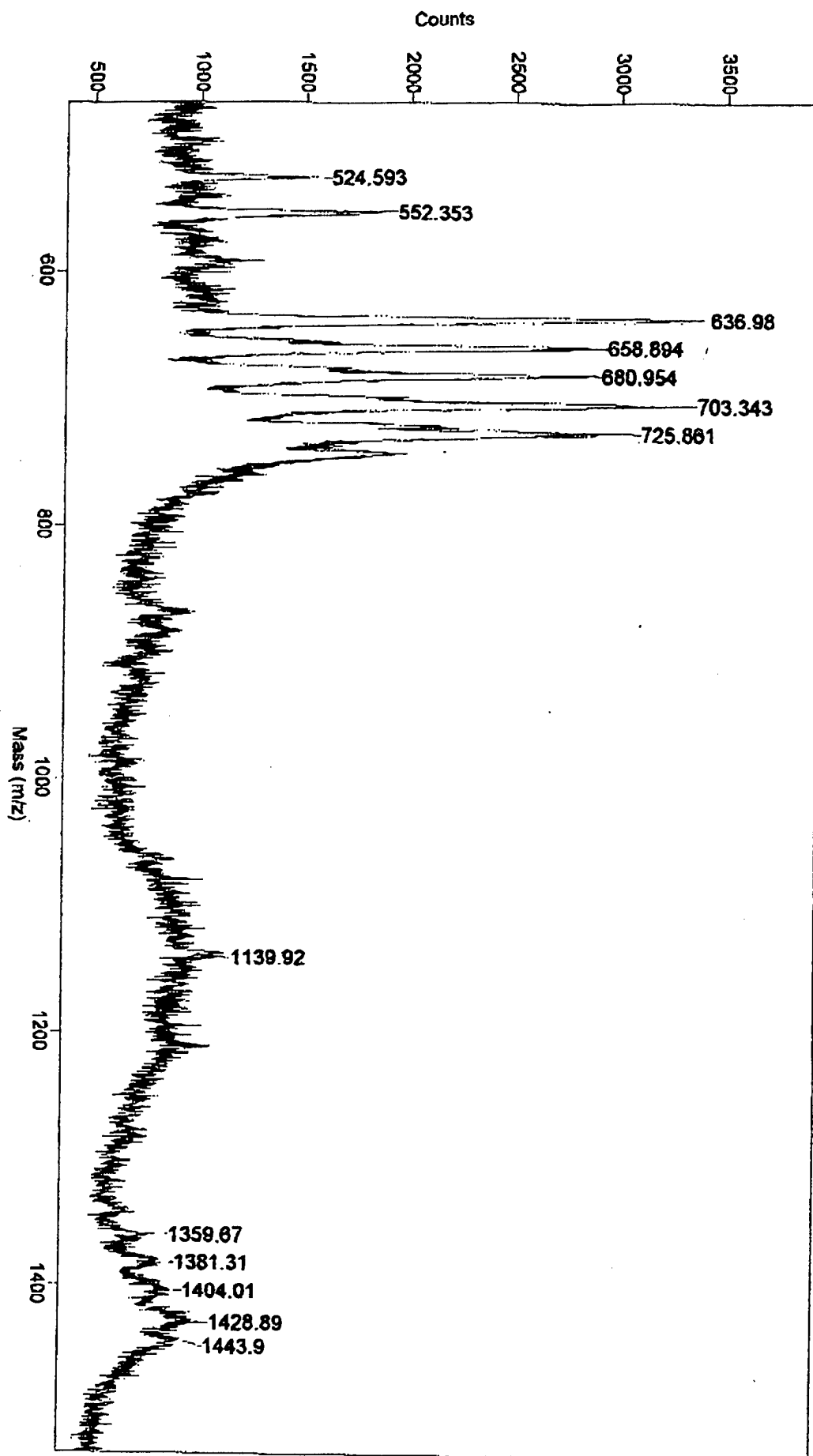
Media   Nickel beads

Figure 1

Figure 2

Figure 3

Tufts Protein Chemistry Facility

SAMPLE: L2

Counts

524.593
552.353
636.98
658.894
680.954
703.343
725.861
1139.92
1359.67
1381.31
1404.01
1428.89
1443.9

Mass (m/z)

Figure 4

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Tufts Protein Chemistry Facility

SAMPLE: AJ2

Figure 5